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(57) Abstract

The use of acidified nitrite as an agent to produce local production of nitric oxide at the skin surface is described in the treatment of peripheral ischaemia and associated conditions. The dosage form may be in any pharmaceutically acceptable carrier means and comprises an acidifying agent adapted to reduce the pH at the environment. A barrier consisting of a membrane allows diffusions of the nitrite ions while preventing direct contact of the skin and acifiying agent. Amongst the many potential applications for the invention is the management of chronic skin wounds, peripheral ischaemia conditions such as Raynaud's phenomenon. Compositions and methods of use for these applications are described.

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PHARMACEUTICAL COMPOSITION CONTAINING NITRATE SOURCE AND AN ACIDIFYING AGENT FOR TREATING SKIN ISCHAEMIA

The present invention relates to a new pharmaceutical use of acidified nitrite contained within a delivery system which allows passage of nitric oxide to the skin as a treatment for ischaemic ulceration, to promote wound healing and associated conditions.

Nitric oxide [NO] is a potent vasodilator synthesised and released by vascular endothelial cells and plays an important role in regulating vascular local resistance and blood flow. In mammalian cells, NO is principally produced along with L-citrulline by the enzymatic oxidation of L-arginine. Nitric oxide is also involved in the inhibition of both platelet and leukocyte aggregation and adhesion, the inhibition of cell proliferation, the scavenging of superoxide radicals and the modulation of endothelial layer permeability. Nitric oxide also has been shown to possess anti-microbial properties, reviewed by F. C. Fang (1997) (*J. Clin. Invest.* 99 (12) 2818-2825 (1997)).

A potential therapeutic utility of the anti-microbial properties of NO is described in WO 95/22335. A pharmaceutical composition comprising nitrite in an inert carrier cream or ointment and salicylic acid was used to show killing of cultures containing *E. coli* and *C. albicans*. This activity was further tested against patients with fungal infection of the feet ("Athlete's Foot" or *tidea pedis*) and showed that the condition was amenable to treatment with the acidified nitrite composition. However, the composition of nitrite and organic acid caused erythema (redness) of the skin.

In addition to internal cell-mediated production, NO is also continually released externally from the surface of the skin by a mechanism which appears to be independent of NO synthase enzyme. Nitrate excreted in sweat is reduced to nitrite by an unknown mechanism which may involve nitrite reductase enzymes which are expressed by skin commensal bacteria. Alternatively mammalian nitrite reductase enzymes may be present in the skin which could reduce nitrite rapidly to NO on the skin surface.

The production of NO from nitrite is believed to be through the following mechanism:

$NO_2^- + H^+ \Leftrightarrow HNO_2$.....[1]

$2HNO_2 \Leftrightarrow N_2O_3 + H_2O$.....[2]

$N_2O_3 \Leftrightarrow NO + NO_2$.....[3]

Although the amount of NO generated by this physiological mechanism is not sufficient to affect skin blood flow it is clear that very large amounts of NO can be generated by the topical application of nitrite and acid.

It has now been surprisingly found that topical application to the skin of nitrite at concentrations of up to 4% in an inert carrier cream or ointment when mixed with an organic acid such as ascorbic acid (vitamin C) reacts to produce oxides of nitrogen to cause the release of nitric oxides leading to sustained vasodilation of the microcirculatory blood vessels, without significant inflammation. This new use for acidified compositions containing nitrite is a departure from the previously known uses of the composition as an anti-microbial agent. The side-effects of erythema and irritation to the skin from the acid in the composition associated with the treatment of fungal infections of the foot had been considered to suggest that the composition should not be used on broken skin or away from sites of infection needing immediate, short term therapeutic treatment. Additionally, the skin on the foot is significantly thicker and tougher than elsewhere on the mammalian body and so can endure more prolonged erythema than other thinner areas of skin elsewhere. Furthermore there is a widespread and generally accepted medical prejudice against inserting ointments or gels into open wounds or onto broken skin. Such practice is advised against because of the risk of actually causing infection or septicaemia (blood-poisoning).

The ability of the composition to cause vasodilation is also surprising because the NO molecule would not normally be expected to cross the outer layers of the skin

into the inner layers of the epidermis to act on the blood vessels and micro-capillaries.

According to a first aspect of the invention there is provided the use of a pharmacologically acceptable acidifying agent, a pharmacologically acceptable source of nitrite ions or a nitrite precursor therefore in the preparation of an agent for the treatment of skin ischaemia and associated conditions.

The pharmacologically acceptable acidifying agent is adapted to reduce the pH at the site of application and can include any suitable organic acid such as ascorbic acid (vitamin C), salicylic acid, acetyl salicylic acid, acetic acid or a salt or a derivative thereof in a concentration up to 20% w/w, suitably 0.25 to 10% w/w, preferably 4 to 6% w/w. A particularly preferred concentration is 4% or 5% w/w. The preferred pH range is from pH2 to pH7, preferably pH4. Other acidifying agents include but are not limited to, ammonium or aluminium salts, phenol, benzoic acid. Inorganic acids such as hydrochloric acid may be used if sufficient dilute and/or appropriately buffered. The acidifying agent may be present as a dissolved salt or in a liquid form.

The pharmacologically acceptable source of nitrite ions may an alkaline metal nitrite or an alkaline earth metal nitrite. For example, $LiNO_2$, $NaNO_2$, KNO_2, $RbNO_2$, $CsNO_2$, $FrNO_2$, $Be(NO_2)_2$, $Mg(NO_2)_2$, $Ca(NO_2)_2$, $Sr(NO_2)_2$, $Ba(NO_2)_2$, or $Ra(NO_2)_2$. Alternatively, a nitrite precursor may be used as the source of the nitrite ions in the composition, such as for example a dilute solution of nitrous acid. Other sources of nitrite ions are nitrate ions derived from alkali metal or alkaline earth metal salts capable of enzymic conversion to nitrite. For example, $LiNO_3$, $NaNO_3$, KNO_3, $RbNO_3$, $CsNO_3$, $FrNO_3$, $Be(NO_3)_2$, $Mg(NO_3)_2$, $Ca(NO_3)_2$, $Sr(NO_3)_2$, $Ba(NO_3)_2$, or $Ra(NO_3)_2$. The concentration of the nitrate ion source may be up to 20% w/w, suitably 0.25 to 10%, preferably 4 to 6%. A particularly preferred concentration is 4% or 5% w/w.

Suitably, the final nitrite ion concentration present in the composition is up to 20%

w/w, generally in the range of from 0.25% to 15% w/w, suitably 2% to 10% w/w, preferably 4 to 6% w/w. A particularly preferred nitrite ion concentration is 4% or 5% w/w.

Ischaemia is defined as an inadequate or impaired blood flow to a part of the body. The present invention seeks to provide the use of a composition in the treatment of skin ischaemia and its associated peripheral skin conditions. For example, disease conditions such as Raynaud's phenomenon and severe primary vasospasm are characterised by poor blood flow to the skin. Damage to the skin of an individual also leads to skin ischaemia as the blood supply is reduced or prevented by the body's own repair or defence mechanisms.

Ischaemic skin conditions which may benefit from the therapeutic use of a composition as defined in accordance with this aspect of the invention, include but are not limited to wounds, including skin ulcers and post-operative trauma, burns. This aspect of the invention therefore also extends to platelet and/or leukocyte aggregation and adhesion, cell proliferation, scavenging of superoxide radicals and endothelial layer permeability. Other dermatological conditions such as acne associated with skin ischaemia can also be treated by these compositions.

In the preparation of an agent according to this aspect of the invention, the acidifying agent and the nitrite ions or source therefore are formulated in a pharmacologically acceptable carrier or diluent which may be an inert cream or ointment. In a particular preferred form of the invention the acidifying agent and the source of nitrite ions or precursor therefore are separately disposed in the said cream or ointment for admixture to release ions at the environment of use.

The pharmaceutical composition may be adapted for administration by any appropriate topical route, including buccal, sublingual or transdermal. Such compositions may be prepared by any method known in the art of pharmacy, for example by admixing the active ingredient with the carrier(s) or excipient(s) under sterile conditions.

Pharmaceutical compositions adapted for transdermal administration may be presented as discrete patches intended to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. For example, the active ingredient may be delivered from the patch by iontophoresis as generally described in *Pharmaceutical Research*, **3(6)**:318 (1986).

Pharmaceutical compositions adapted for topical administration may be formulated as ointments, creams, suspensions, lotions, powders, solutions, pastes, gels, sprays, aerosols or oils. For treatment of the eye or other external tissues, for example mouth and skin, the compositions are preferably applied as a topical ointment or cream. When formulated in an ointment, the active ingredient may be employed with either a paraffinic or a water-miscible ointment base. Alternatively, the active ingredient may be formulated in a cream with an oil-in-water cream base or a water-in-oil base. Pharmaceutical compositions adapted for topical administration to the eye include eye drops wherein the active ingredient is dissolved or suspended in a suitable carrier, especially an aqueous solvent. Pharmaceutical compositions adapted for topical administration in the mouth include lozenges, pastilles and mouth washes.

The pharmaceutical compositions may contain preserving agents, solubilising agents, stabilising agents, wetting agents, emulsifiers, sweeteners, colourants, odourants, salts (substances of the present invention may themselves be provided in the form of a pharmaceutically acceptable salt), buffers, coating agents or antioxidants. They may also contain therapeutically active agents in addition to the substance of the present invention.

Dosages of the substance of the present invention can vary between wide limits, depending upon the disease or disorder to be treated, the severity of the condition, and the age and health of the individual to be treated, etc. and a physician will ultimately determine appropriate dosages to be used.

This dosage may be repeated as often as appropriate. If side effects develop the amount and/or frequency of the dosage can be reduced or otherwise altered or modified, in accordance with normal clinical practice.

Such compositions may be formulated for human or for veterinary medicine. The present application should be interpreted as applying equally to humans as well as to animals, unless the context clearly implies otherwise.

According to a second aspect of the invention there is provided a method for the treatment of a condition characterised by skin ischaemia, comprising the administration of a composition comprising a pharmacologically acceptable acidifying agent, a pharmacologically acceptable source of nitrite ions or a nitrite precursor therefore.

According to a third aspect of the invention there is provided a composition comprising a pharmacologically acceptable acidifying agent, a pharmacologically acceptable source of nitrite ions or a nitrite precursor therefore as a combined preparation for simultaneous, separate or sequential use in the treatment of skin ischaemia.

According to a fourth aspect of the invention there is provided a kit comprising a pharmacologically acceptable acidifying agent and a pharmacologically acceptable source of nitrite ions or a nitrite precursor therefore for use as a combined preparation in the treatment of skin ischaemia

According to a fifth aspect of the present invention there is provided a membrane comprising a pharmacologically acceptable acidifying agent and a pharmacologically acceptable source of nitrite ions or a nitrite precursor therefore. The membrane may be fully-, or partially-permeable, including semi-permeable or selectively permeable, to the passage of nitric oxide. Such membranes can prevent direct contact of the composition with the skin but can permit diffusion of nitric oxides into the skin.

This is particularly advantageous in the treatment of areas of broken skin, open wounds or serious burns. In this way the integrity of the wound area is preserved. Suitable membranes include, but are not limited to, polymeric materials such as nitrocellulose, cellulose, agarose, alginate gels, polyethylene, polyester (e.g. a hydrophilic polyester block copolymer) etc. A suitable membrane that can be used in practice is Sympatex™ which is composed of fibres of hydrophilic polyester block copolymer. The present invention therefore extends to the use of such membranes in the treatment of these and other disease conditions, for example skin ischaemia and/or microbial infections, e.g. bacterial, yeast or fungal infections.

Preferred features for the second and subsequent aspects of the invention are as for the first aspect *mutatis mutandis*.

The invention will now be described, by way of illustration only with reference to the following examples and figures which are provided for the purposes of illustration and are not to be construed as being limiting on the invention.

FIGURE 1 shows the effect of direct application and subsequent removal of the treatment on the microcirculatory blood flow in forearm skin and finger pulps of healthy subjects. The vertical axes are blood flow, photoplethysmography (PPG) relating to microcirculatory volume and laser Doppler fluximetry (LDF) which relates relating to microcirculatory flux (red blood cell count x velocity). The horizontal axis is the time in minutes; NS = not significant; points shown represent the mean value; error bars are 95% confidence; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; ↑ = application of gel, and ↓ = removal of gel.

FIGURE 2 shows the effect of direct application and subsequent removal of the treatment on the microcirculatory blood flow in forearm skin and finger pulps of subjects with severe Raynaud's phenomenon. The vertical axes are blood flow, photoplethysmography (PPG) relating to microcirculatory volume

and laser Doppler fluximetry (LDF) which relates to microcirculatory flux. The horizontal axis is the time in minutes.

FIGURE 3 shows nitric oxide diffusion through a selection of membranes where the vertical axis shows nitric oxide concentration and the horizontal axis in the time in minutes. FIGURE 3a shows the results using Saranwrap™ (SW-01) and FIGURE 3b shows the results using clingfilm (CF-02).

FIGURE 4 shows the diffusion effect of the treatment through a membrane on the forearm skin microcirculatory blood flow in a healthy subject. The vertical axis is blood flow, photoplethysmography (PPG) relating to microcirculatory volume and the horizontal axis is the time in minutes.

FIGURE 5 shows the diffusion effect of the treatment through a membrane on forearm skin microcirculatory blood flow in a healthy subject. The vertical axis is blood flow, laser Doppler fluximetry (LDF) relating to microcirculatory flux and the horizontal axis is the time in minutes.

FIGUREs 6 (a)-(i) show the transmembrane diffusion for sodium nitrite and ascorbic acid in 0.8% agar gel, using 1% sodium chloride as an intermediate at final concentrations of 500mM, 250mM, 165mM, 50mM, 25mM, 5mM, 2.5mM and 0.5mM. A control of nitrite and 0.8% agar gel using 1% sodium chloride as an intermediate was also used. The figure illustrates nitric oxide diffusion through Sympatex™ 10μm (Akzo Nobel) membrane where the vertical axis shows the nitric oxide concentration in parts per million (PPM) and the horizontal axis shows the time in minutes. In Figures 6(a) and 6(b) the initial peaks are artificially flattened due to the full scale deflection of the detection device.

FIGURE 7 shows the results of the application of nitric oxide generating gel consisting of 330mM of sodium nitrite and ascorbic acid in KY jelly™ to the forearm skin and simultaneously to SympatexTM 10μm membrane (Akzo

Nobel), which was then applied to the forearm skin of the contralateral limb if nine healthy subjects. Conditions and experimental methods were the same as used for the application of the NO-generation gel on healthy subjects in Figures 1, 2, 4 and 5. The vertical axis shows Laser Doppler Fluximetry units and the horizontal axis shows the time in minutes.

FIGURE 8 shows the anti-microbial properties of the NO-generation gel at different nitrite ion concentrations against *Staphylococcus aureus* NCTC9353 and *Escherichia coli* NCTC10148. The vertical axis shows microbial survival as a percentage and the horizontal axis shows NO-gel concentration in mM.

Example 1: Microcirculatory response to topical application of NO-generating gel in healthy subjects

A nitric oxide-generating gel (NO-generating gel) was prepared as follows. Sodium nitrite (Analar™ grade from Sigma, Poole, Dorset, UK) was added to KY Jelly™ (Johnson & Johnson) to make a 5% w/w solution. Ascorbic acid (Sigma) was also added to KY Jelly™ (Johnson & Johnson) to make a 5% w/w solution. Approximately 0.5ml of each solution was mixed together on the skin of a patient using a sterile swab. When the two solutions are brought into contact, the ensuing reaction leads to the generation of nitric oxide. The reaction may be stopped by cleaning the skin with paper or a swab soaked in ethyl alcohol.

With reference to Figure 1 the microcirculatory response to topical application of NO-generating gel was measured in 10 healthy subjects. The effect of placebo treatment was measured simultaneously on the contra-lateral limb. The skin microcirculatory volume was measured by infra-red photoplethysmography [PPG] and microcirculatory velocity by laser Doppler fluximetry [LDF]. All examinations were performed in a quiet, draught-free, temperature and humidity controlled laboratory (24°C ± 1°C; relative humidity 30-40%) in the morning at approximately the same time of day for each subject.

Placebo treatment did not have any effect upon microcirculatory blood flow in either the forearm or the finger of the normal subjects. The vasodilator response to the active treatment reached a plateau phase in all patients within the ten minutes of active gel application. Forearm skin and finger pulp blood flow increased markedly following topical application of a NO-generating gel in the healthy volunteers. When the active gel was applied to the forearm skin all subjects showed a large vasodilator response to active gel treatment in both volume and flux. This increase in blood flow was sustained after removal of the active gel. The active gel had no significant effect on finger microcirculatory volume (PPG) (Figure 1 : Finger pulp), however microcirculatory flux increased significantly ($p<0.01$) and remained so after removal ($p<0.01$; Figure 1 : Finger pulp).

Example 2: Microcirculatory response to topical application of NO-generating gel in patients with severe primary vasospasm

Figure 2 shows the microcirculatory response to topical application of NO-generating gel was measured in 20 patients with severe primary vasospasm. The effect of the placebo treatment was measured simultaneously on the contra-lateral limb. Conditions were the same as those used for the application of the treatment on healthy subjects in Figure 1. The skin microcirculatory volume was measured by infra-red photoplethysmography [PPG] and microcirculatory velocity by laser Doppler fluximetry [LDF].

Placebo treatment did not have any effect upon microcirculatory blood flow in either the forearm or the finger of any patients. The vasodilator response to the active treatment reached a plateau phase in all patients within ten minutes of the application of active gel. When the gel was applied to the forearm skin all patients showed a large vasodilator response to active gel treatment in both volume and flux. This increase in blood flow was sustained after removal of the active gel in both groups (Figure 2 : Forearm and finger pulp). The active gel to the finger pulp caused a significant increase in microcirculatory volume ($p<0.05$) which returned rapidly to the resting level on removal of the gel. Active gel also significantly increased finger microcirculatory flux ($p<0.01$) which achieved normal values. This increase was

sustained, although reduced, after removal of the gel ($p<0.05$).

Example 3: Generation of nitric oxide derived through a membrane

Figure 3 shows the generation of nitric oxide derived from the reaction previously detailed through a membrane. Nitric oxide concentrations were measured by a nitric oxide sensitive meter : Model 42C Chemiluminescence NO-NO_2-NO_x analyser (Thermo Environmental Instruments Inc., MA USA) connected to a data acquisition system and IBM computer. Measurements were made continually and readings were taken every 10 seconds for 275 minutes. Material 1 was domestic clingfilm, Material 2 was Saranwrap™ (Sigma) and Material 3 was (Sympatex™, Akzo Nobel).

Example 4: Microcirculatory response of the application of NO-generating gel to three differing membrane materials

Figure 4 shows the microcirculatory response of the application of NO-generating gel to three differing membranes which were then applied to the forearm skin of a healthy subject. Conditions were the same as those used for the application of the treatment upon healthy subjects in Figure 1. The skin microcirculatory volume was measured by infra-red photoplethysmography [PPG]. Material 1 was domestic clingfilm, Material 2 was Saranwrap™ (Sigma) and Material 3 was (Sympatex™, Akzo Nobel).

The increase in microcirculatory blood volume is a reflection of the diffusion of nitric oxide through the membrane towards the skin. The transfer of nitric oxide through the membrane is a reflection of the physical characteristics of the material and is highly variable. Material number 3 (Sympatex™, Akzo Nobel) had a superior diffusion profile.

Example 5: Microcirculatory response of the application of NO-generating gel to three differing membrane materials

Figure 5 shows the microcirculatory response of the application of NO-generating gel to three differing membranes which were then applied to the forearm skin of a healthy subject. Conditions were the same as those used for the application of the

treatment on healthy subjects in Figure 1. The skin microcirculatory velocity was measured by laser Doppler fluximetry [LDF].

The increase in microcirculatory velocity is a reflection of the diffusion of nitric oxide through the membrane towards the skin. The transfer the nitric oxide through the membrane is a reflection of the physical characteristics of the material and is highly variable. Material number 3 (Sympatex™, Akzo Nobel) had a superior diffusion profile.

Example 6: Comparison of nitric oxide generation through a membrane

Figure 6 shows the generation of nitric oxide derived from the reaction described above through a 10μm Sympatex™ membrane. Nitric oxide concentrations were measured by a nitric oxide sensitive meter: Model 42C chemiluminescence NO-NO_2-NO_x analyser (Thermo Environmental Instrumental Inc., MA, USA) connected to a data acquisition system and an IBM computer. Measurements were made continually and readings were taken every 10 seconds for 1350 minutes.

The results shown in Figure 6 illustrate that the transmembrane diffusion coefficient is closely related to the production of nitric oxide, which is a direct product of the concentration of both the source of the nitrite ions and the acidifying agent. Furthermore, the results demonstrate that a basal production of nitric oxide is sustained for a significant period of time after mixing the reagents.

Example 7: Microcirculatory response of the application of NO-generating gel

The nitric oxide generating gel consisting of 330mM of both sodium nitrite and ascorbic acid in KY jelly™ was applied directly to the forearm skin and simultaneously to SympatexTM 10μm membrane (Akzo Nobel), which was then applied to the forearm skin of the contralateral limb if nine healthy subjects. Conditions and experimental methods were the same as used for the application of the NO-generation gel on healthy subjects in Figures 1, 2, 4 and 5. The results are shown in Figure 7. It should be noted that in Figure 7 that the concentrations of the admixture are in a different unit form (i.e. mM instead of %w/w). Laser Doppler

Fluximetry (LDF) measured the skin microcirculatory flux.

The statistically significant increase in microcirculatory flux from baseline was a reflection of the diffusion of nitric oxide through the membrane towards the skin. This vasodilation, indicated by LDF through the membrane ranged from 60-75% (mean 64%) of that observed when the NO-generation gel was applied directly to the skin of the forearm. The results shown in Figure 7 support the observations described in Figure 1 which show that the vasodilator response to the direct treatment reached a plateau phase in all patients within 10 minutes of gel application. A plateau phase, although reduced in amplitude was achieved within 16 minutes when the NO-generation gel was applied to the membrane and reflects a lag phase which is related to membrane diffusion characteristics.

Example 8: Anti-microbial properties of NO-generation gel

The antimicrobial properties of NO-generation gel after diffusion through a 10μm Sympatex™ membrane were investigated as follows. NO was generated by an admixture of sodium nitrite and ascorbic acid in 0.8% agar gel, using 1% sodium chloride as an intermediate. The preparation was tested on *S. aureus* NCTC9353 and *E. coli* NCTC10148 using a range of concentrations of sodium nitrite and ascorbic acid. Cultures of *S. aureus* and *E. coli* were prepared by innoculating 20ml of LB (Luria-Bertani 10g Bacto-Tryptone, 5g Bacto-Yeast extract and 10g/l sodium chloride at pH7.5) broth with 2-3 colonies, and incubated at 37°C overnight. 24ml of 1.5% agar in NaCl were innoculated with 1ml of either *S. aureus* or *E. coli* and poured into Petri dishes. Discs of membrane (100mm in diameter) were sterilised in 70% ethanol and the discs were then placed in a lamina flow cabinet to allow the ethanol to evaporate. 5ml of 0.8% agar in 1% NaCl, containing either sodium nitrite or ascorbic acid at final concentrations of 500mM, 250mM, 165mM, 50mM, 25mM, 5mM, 2.5mM and 0.5mM were prepared. Final concentrations in use are halved.

In the centre of sterile inverted Petri dish lids, 1ml of each concentration of sodium nitrite and ascorbic acid was added and mixed. Disinfected membrane was then

placed over the top of this immediately, using sterilised forceps. The membrane was carefully positioned so that it hung over the edge of the lid equally in all directions. The base of the Petri dish was then placed upside down on top of the lid/mixture/membrane arrangement ensuring that a 2-3mm gap was left between the membrane and the inverted innoculated agar.

The apparatus was incubated overnight at 37°C after which it was removed. The base of the Petri dish (upside down) was removed and the central area of agar sampled by cutting a circle using a sterile plastic measuring cup. The agar was then macerated in 10ml of LB broth and 5ml of sterile glass beads. Serial dilutions were carried out and the samples plated onto blood agar plates that were incubated for 24 hours at 37°C. The surviving colonies were then counted.

Anti-microbial properties of nitric oxide were seen at concentrations of nitrite above 50mM. Below this concentration partial or no anti-microbial activity was seen. Above this concentration, cell lysis was complete resulting in complete killing of the bacteria. The results shown in Figure 8 illustrate the anti-microbial effect of varying concentrations of NO-generation gel and resulting diffusion through Sympatex™ 10μm membrane.

CLAIMS

1. The use of a pharmacologically acceptable acidifying agent, a pharmacologically acceptable source of nitrite ions or a nitrite precursor therefore in the preparation of an agent for the treatment of skin ischaemia and associated conditions.

2. A use as claimed in claim 1, in which the acidifying agent is an organic acid.

3. A use as claimed in claim 2, in which the organic acid is ascorbic acid.

4. A use as claimed in any one of claims 1 to 3, in which the source of nitrite ions is an alkaline metal nitrite or an alkaline earth metal nitrite.

5. A use as claimed in any one of claims 1 to 5, in which the ischaemic skin disease is Raynaud's phenomenon.

6. A use as claimed in any one of claims 1 to 5, in which the treatment of skin ischaemia includes the treatment of wounds, including skin ulcers and post-operative trauma, or burns.

7. A method for the treatment of a condition characterised by skin ischaemia, comprising the administration of a composition comprising a pharmacologically acceptable acidifying agent, a pharmacologically acceptable source of nitrite ions or a nitrite precursor therefore.

8. A composition comprising a pharmacologically acceptable acidifying agent, a pharmacologically acceptable source of nitrite ions or a nitrite precursor therefore as a combined preparation for simultaneous, separate or sequential use in the treatment of skin ischaemia.

9. A composition comprising a pharmacologically acceptable acidifying agent, a pharmacologically acceptable source of nitrite ions or a nitrite precursor therefore as a combined preparation for simultaneous, separate or sequential use in the treatment of wounds, including skin ulcers and post-operative trauma, or burns.

10. A kit comprising a pharmacologically acceptable acidifying agent and a pharmacologically acceptable source of nitrite ions or a nitrite precursor therefore for use as a combined preparation in the treatment of skin ischaemia

11. A kit comprising a pharmacologically acceptable acidifying agent and a pharmacologically acceptable source of nitrite ions or a nitrite precursor therefore for use as a combined preparation in the treatment of wounds, including skin ulcers and post-operative trauma, or burns.

12. A membrane comprising a pharmacologically acceptable acidifying agent and a pharmacologically acceptable source of nitrite ions or a nitrite precursor therefore.

13. A membrane as claimed in claim 12, in which the acidifying agent is an organic acid.

14. A membrane as claimed in claim 13, in which the organic acid is ascorbic acid.

15. A membrane as claimed in any one of claims 12 to 14, in which the source of nitrite ions is an alkaline metal nitrite or an alkaline earth metal nitrite.

16. The use of a membrane as defined in any one of claims 12 to 15 in the treatment of skin ischaemia.

17. A use as claimed in claim 16, in which the treatment of skin ischaemia includes the treatment of wounds, including skin ulcers and post-operative trauma, or

burns.

18. The use of a membrane as defined in any of claims 12 to 15 in the treatment of microbial infections.

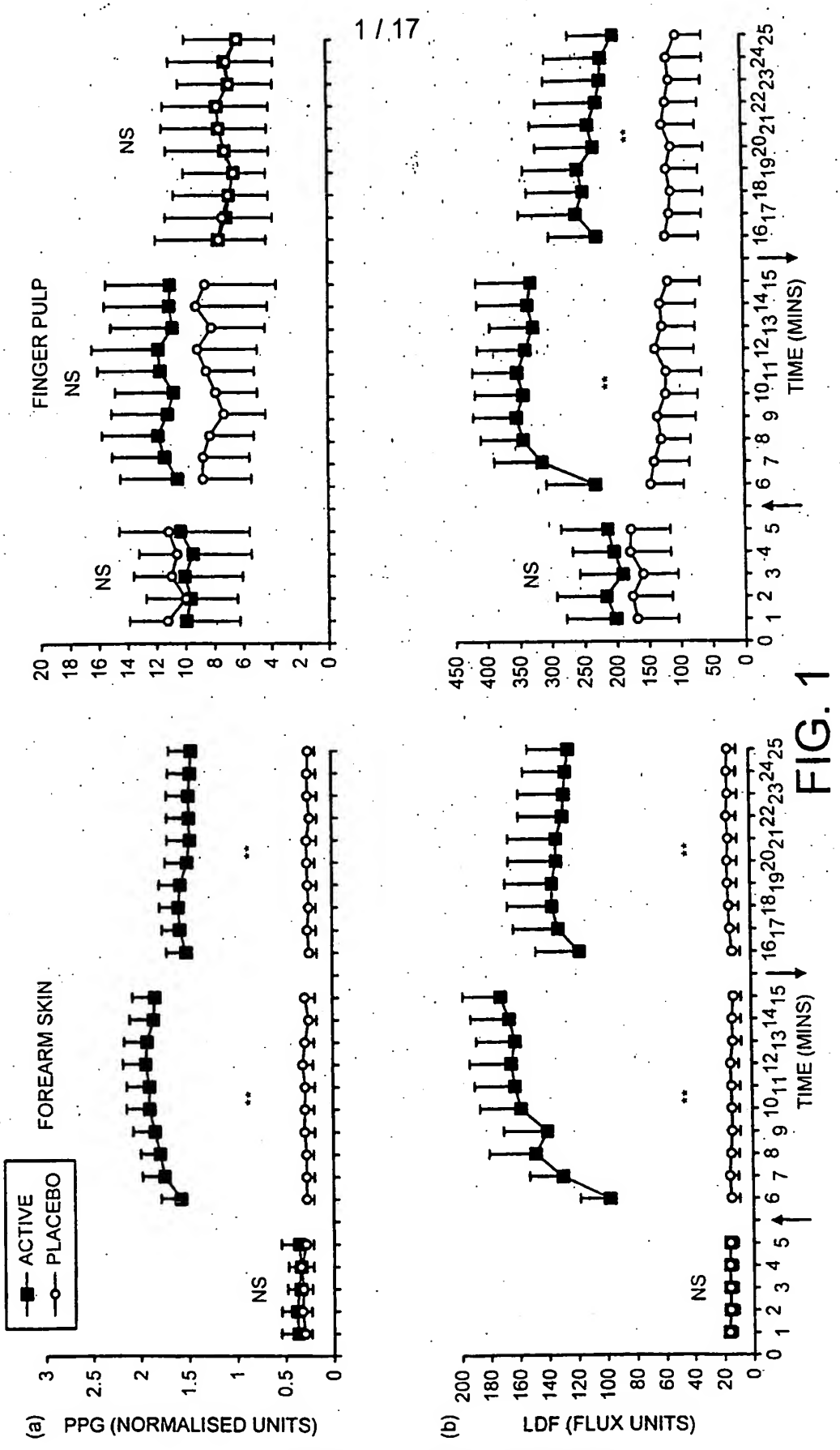
FINGER PULP
FOREARM SKIN
ACTIVE
PLACEBO
NS
**
(a) PPG (NORMALISED UNITS)
(b) LDF (FLUX UNITS)
TIME (MINS)

FIG. 1

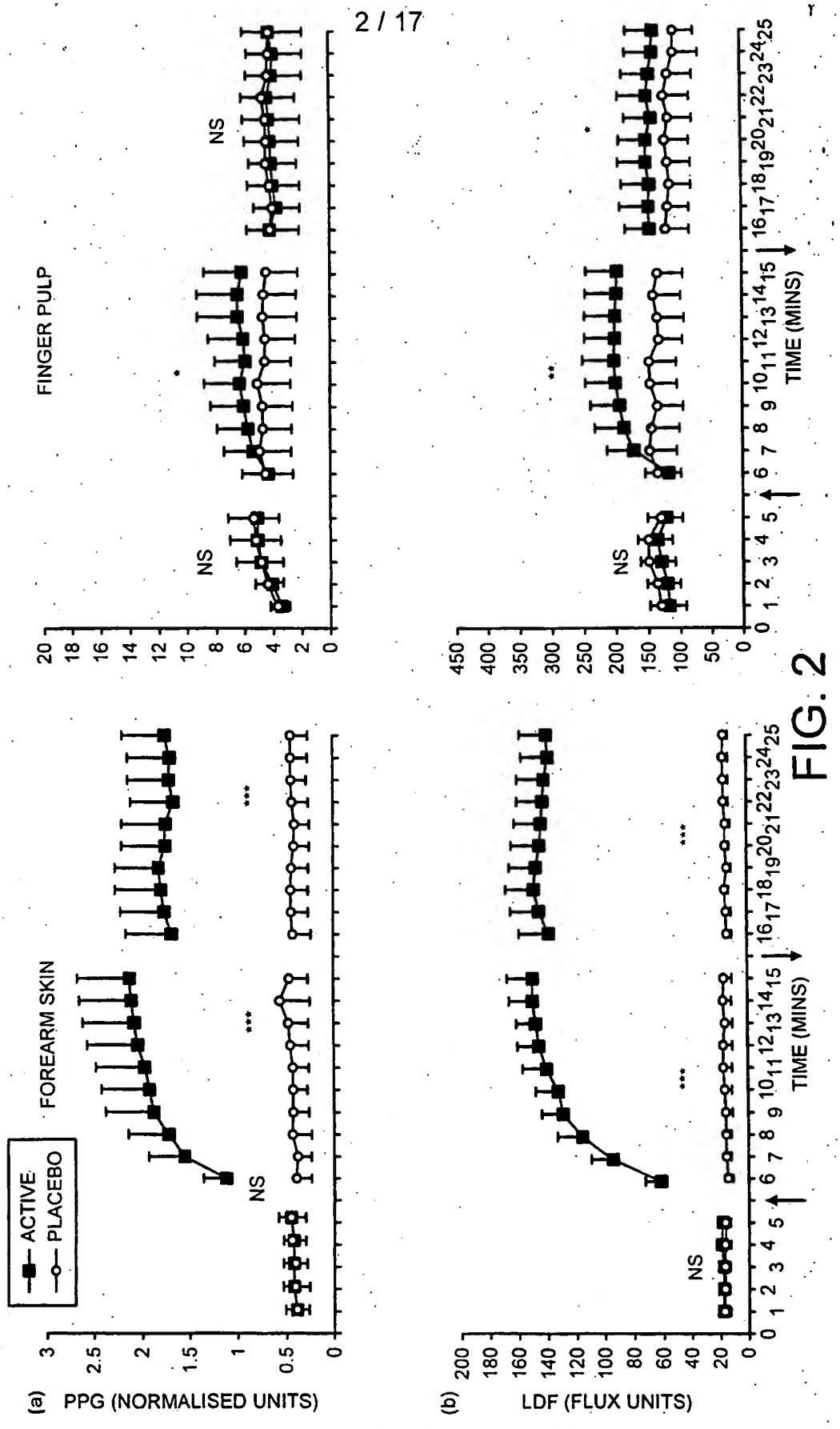
FINGER PULP
FOREARM SKIN
ACTIVE
PLACEBO
(a) PPG (NORMALISED UNITS)
(b) LDF (FLUX UNITS)
TIME (MINS)
NS
***
**

FIG. 2

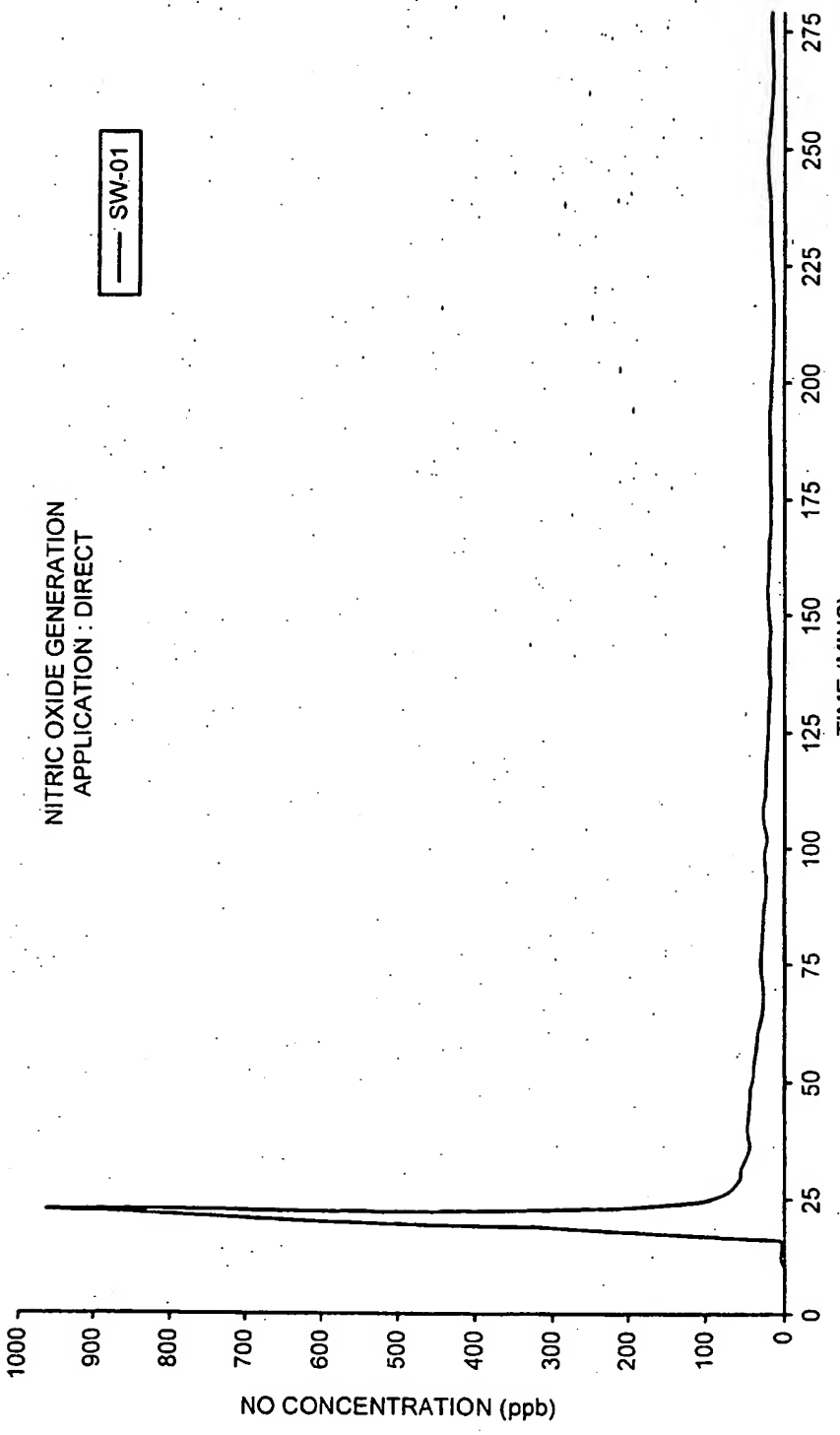
NITRIC OXIDE GENERATION
APPLICATION : DIRECT
SW-01
NO CONCENTRATION (ppb)
1000
900
800
700
600
500
400
300
200
100
0
TIME (MINS)
0
25
50
75
100
125
150
175
200
225
250
275

FIG. 3a

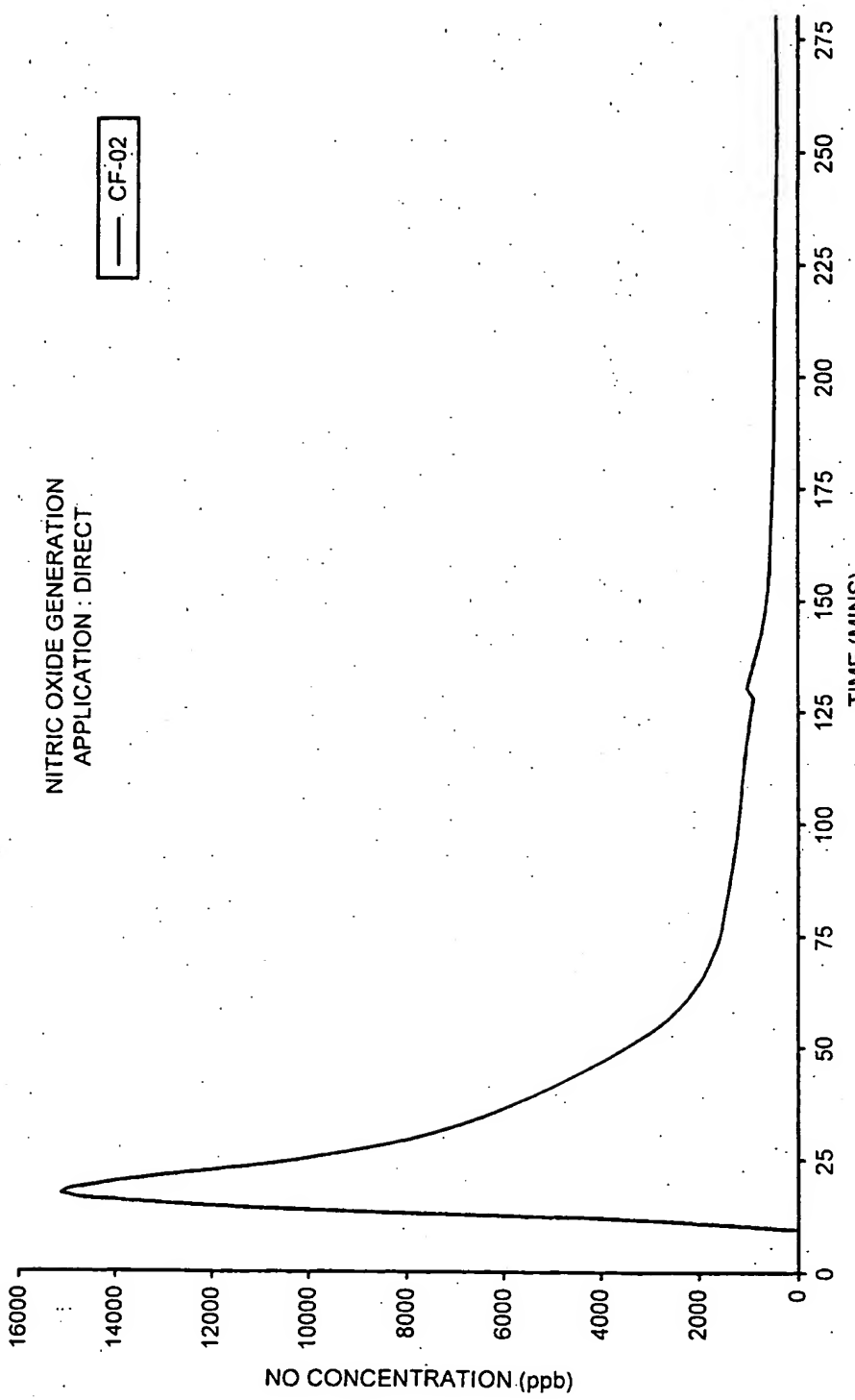
NITRIC OXIDE GENERATION
APPLICATION : DIRECT
CF-02
NO CONCENTRATION (ppb)
16000
14000
12000
10000
8000
6000
4000
2000
0
TIME (MINS)
0
25
50
75
100
125
150
175
200
225
250
275

FIG. 3b

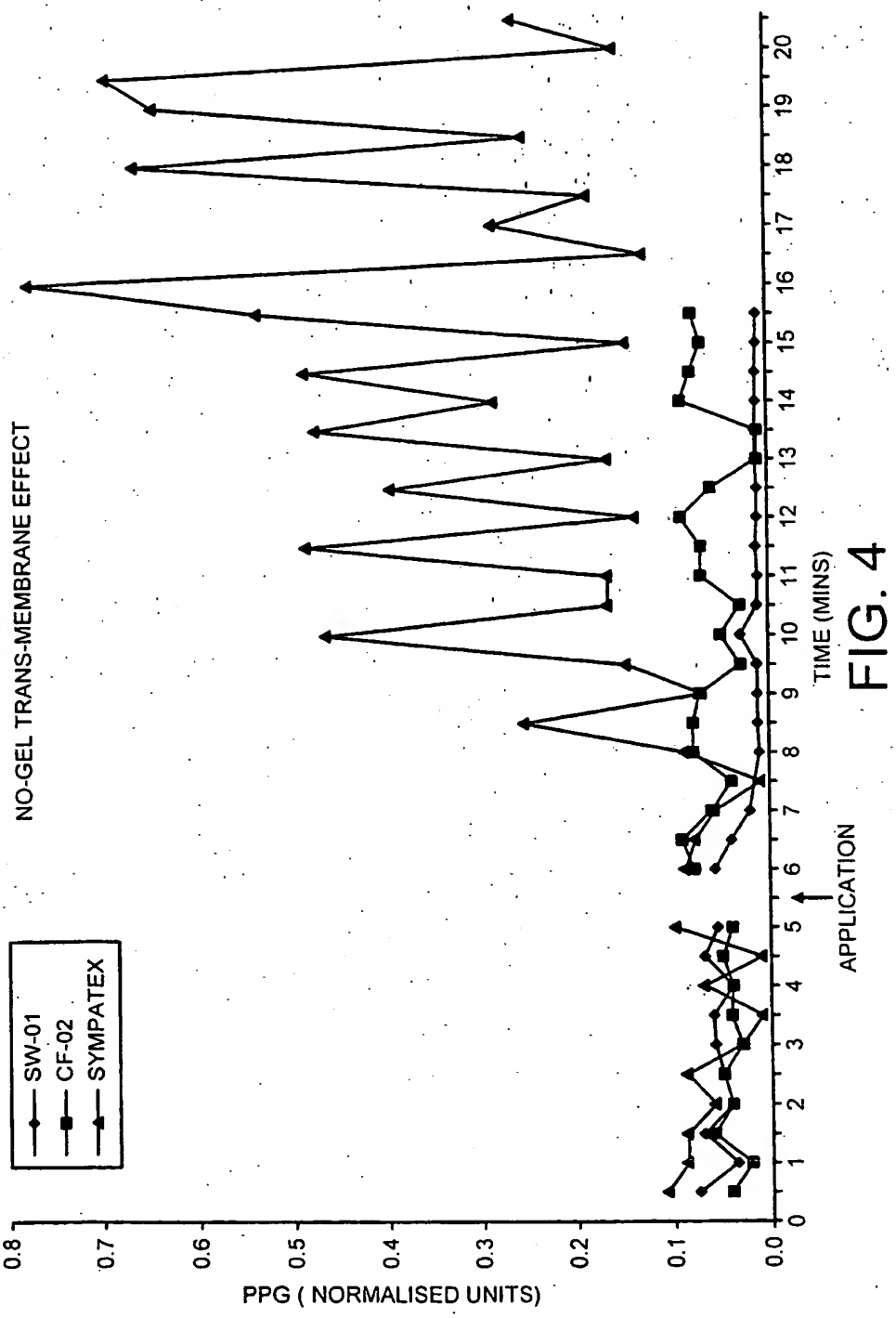
NO-GEL TRANS-MEMBRANE EFFECT
SW-01
CF-02
SYMPATEX
PPG (NORMALISED UNITS)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
TIME (MINS)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
APPLICATION

FIG. 4

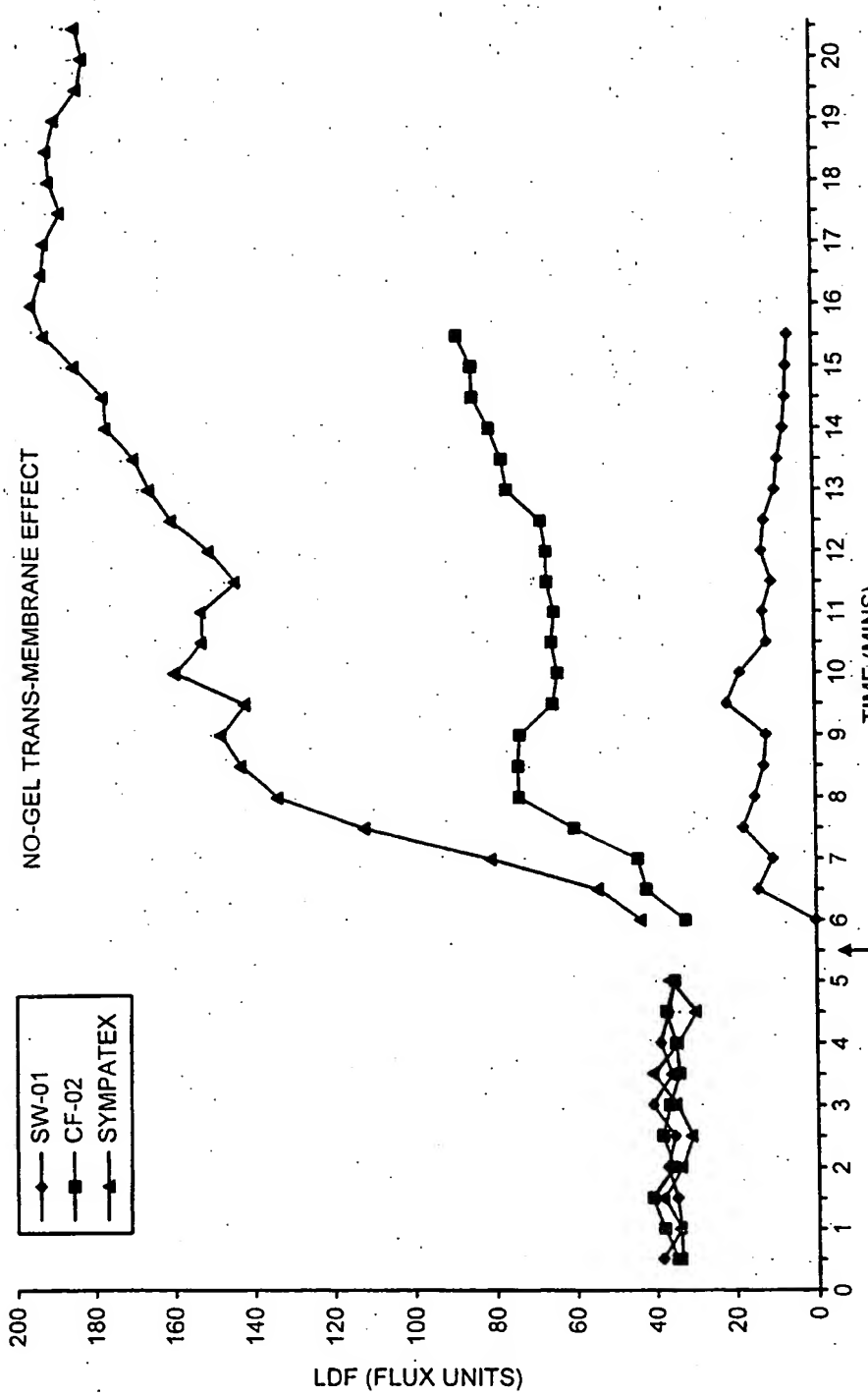
NO-GEL TRANS-MEMBRANE EFFECT
SW-01
CF-02
SYMPATEX
LDF (FLUX UNITS)
200
180
160
140
120
100
80
60
40
20
0
TIME (MINS)
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
APPLICATION

FIG. 5

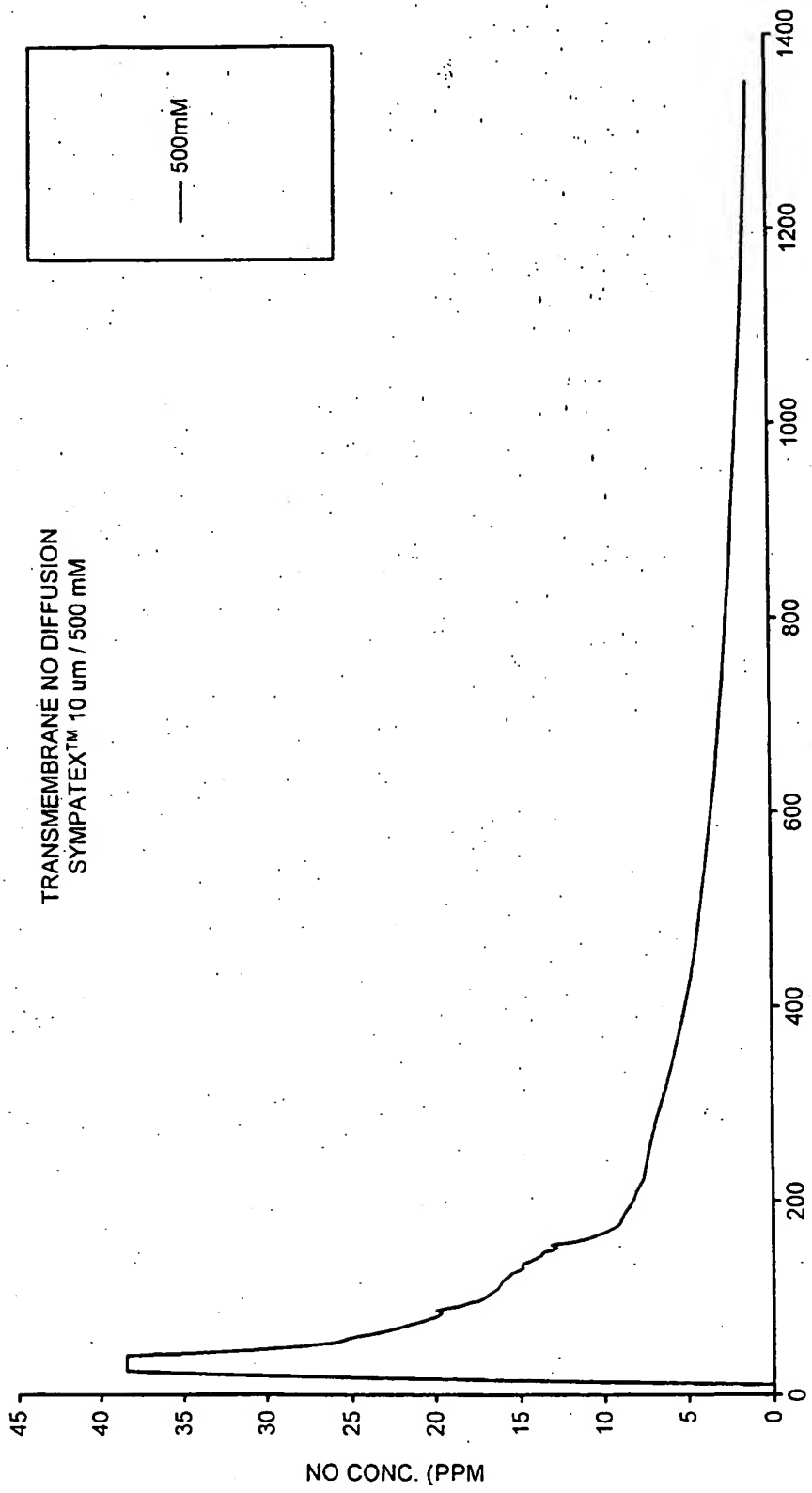
500mM
TRANSMEMBRANE NO DIFFUSION
SYMPATEX™ 10 um / 500 mM
NO CONC. (PPM
0
5
10
15
20
25
30
35
40
45
TIME (MINS)
0
200
400
600
800
1000
1200
1400

FIG. 6a

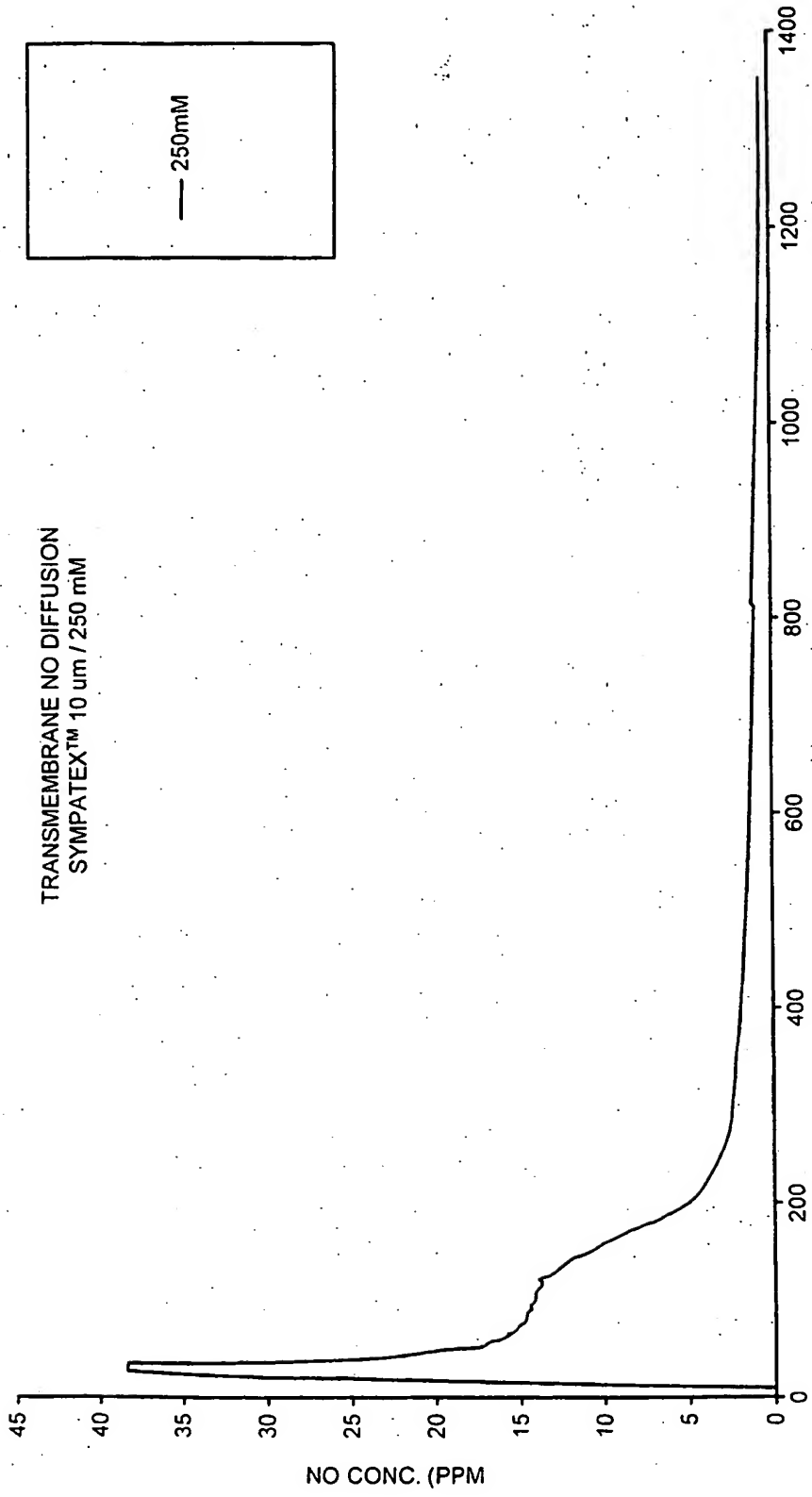
250mM
TRANSMEMBRANE NO DIFFUSION
SYMPATEX™ 10 um / 250 mM
NO CONC. (PPM
TIME (MINS)
0
5
10
15
20
25
30
35
40
45
0
200
400
600
800
1000
1200
1400

FIG. 6b

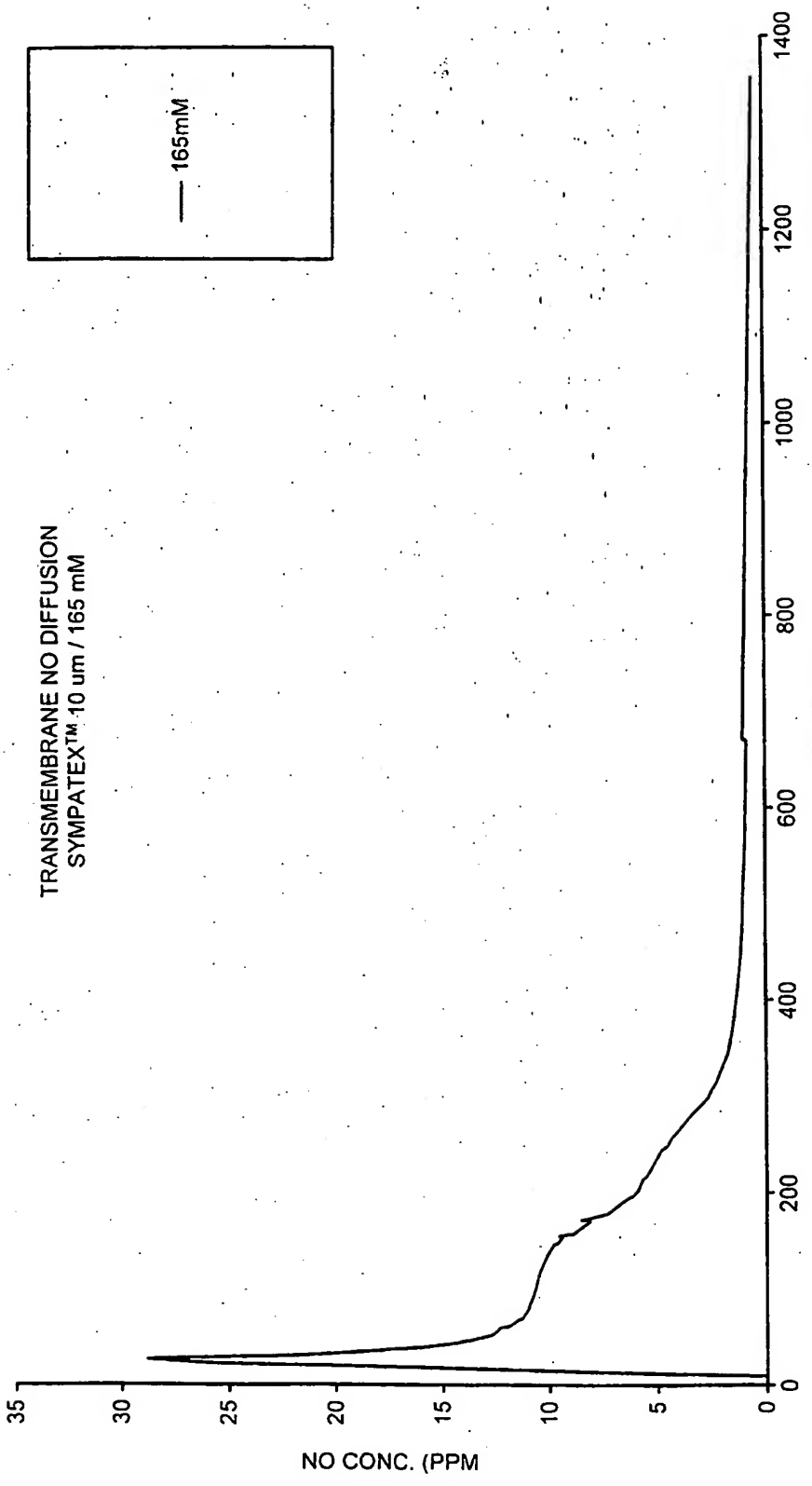
165mM
TRANSMEMBRANE NO DIFFUSION
SYMPATEX™ 10 um / 165 mM
NO CONC. (PPM
TIME (MINS)
0
5
10
15
20
25
30
35
0
200
400
600
800
1000
1200
1400

FIG. 6c

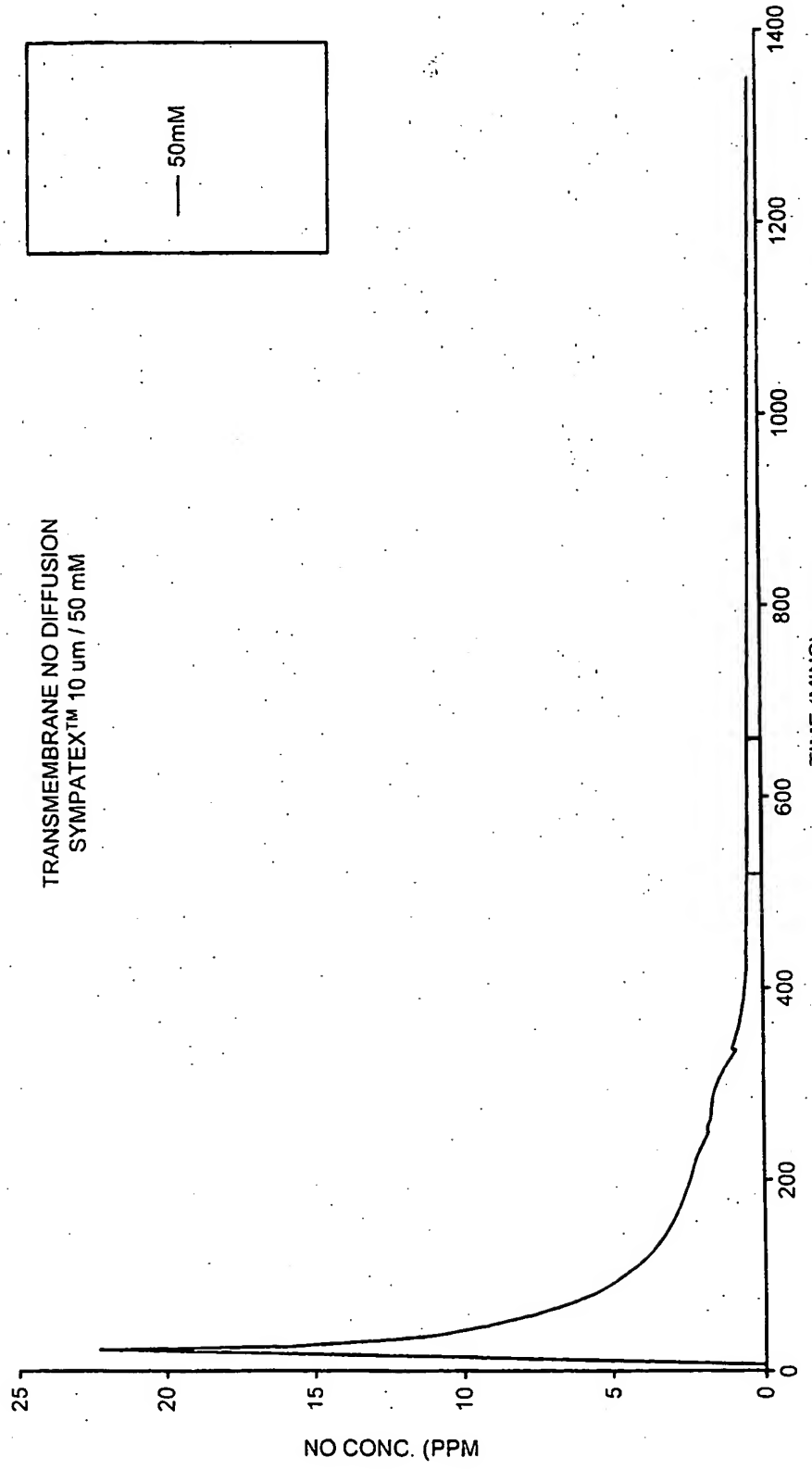
50mM
TRANSMEMBRANE NO DIFFUSION
SYMPATEX™ 10 um / 50 mM
NO CONC. (PPM
0
5
10
15
20
25
TIME (MINS)
0
200
400
600
800
1000
1200
1400

FIG. 6d

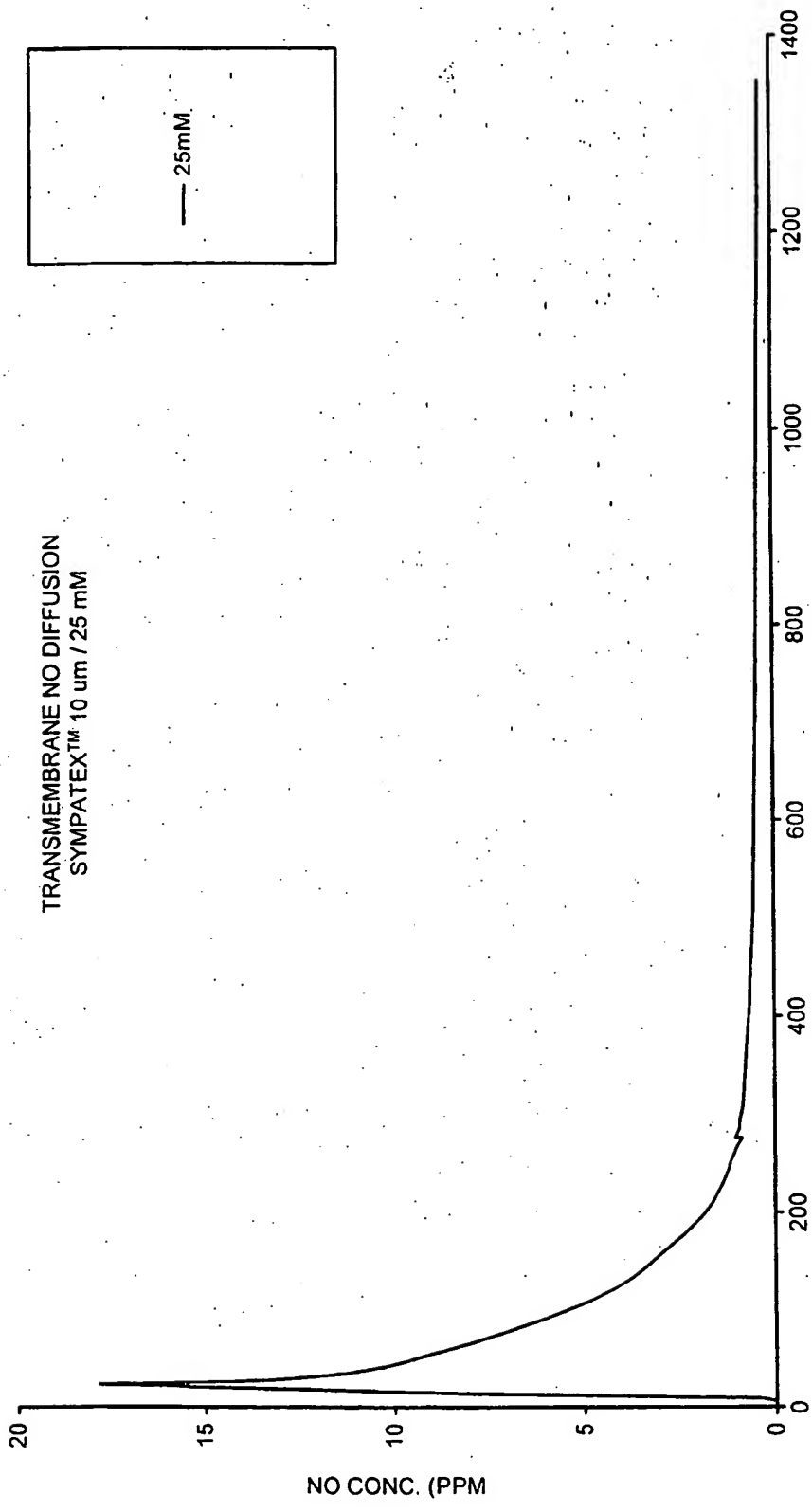
25mM
TRANSMEMBRANE NO DIFFUSION
SYMPATEX™ 10 um / 25 mM
NO CONC. (PPM
20
15
10
5
0
TIME (MINS)
0
200
400
600
800
1000
1200
1400

FIG. 6e

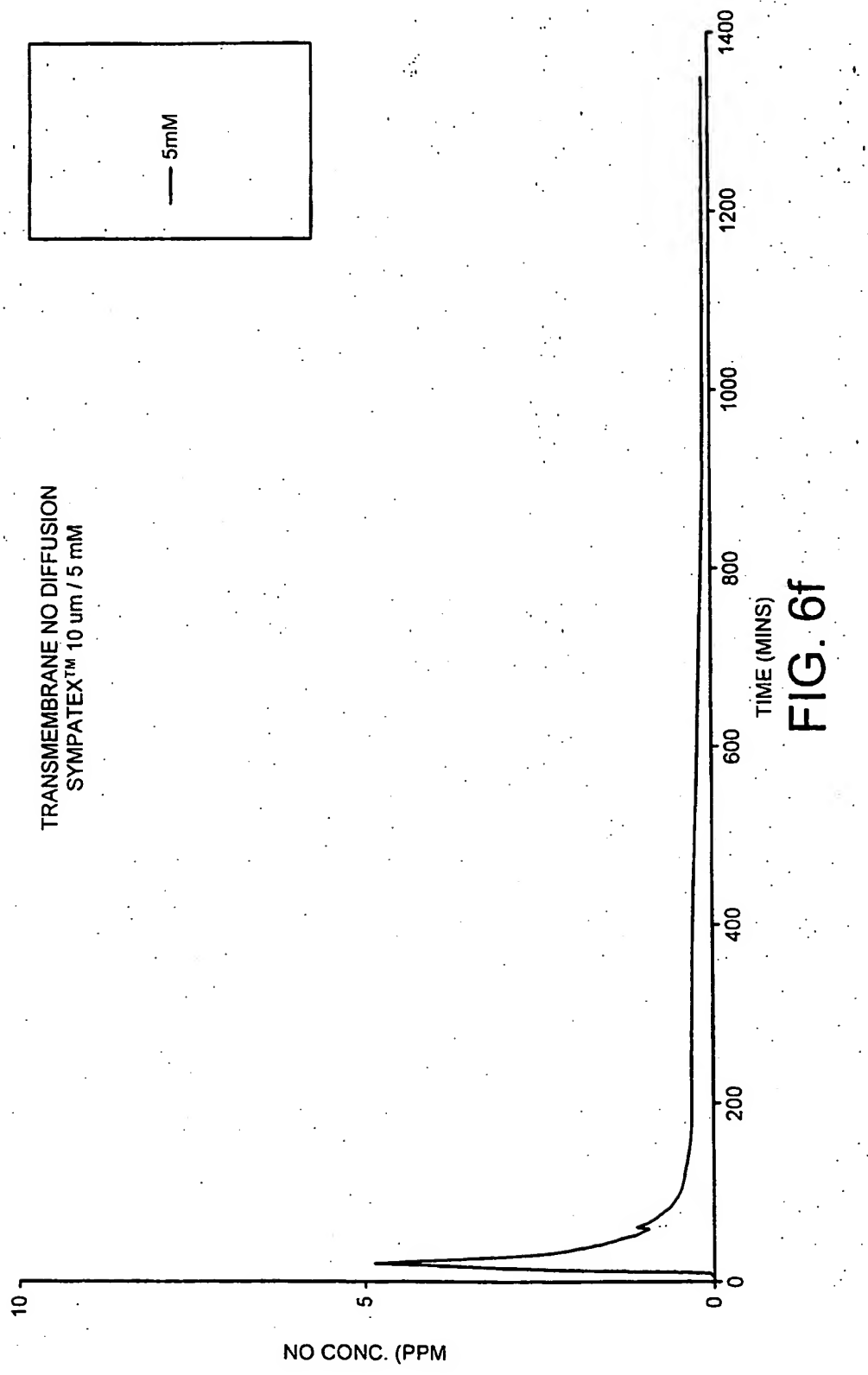
5mM
TRANSMEMBRANE NO DIFFUSION
SYMPATEX™ 10 um / 5 mM
TIME (MINS)
0
200
400
600
800
1000
1200
1400
NO CONC. (PPM
0
5
10

FIG. 6f

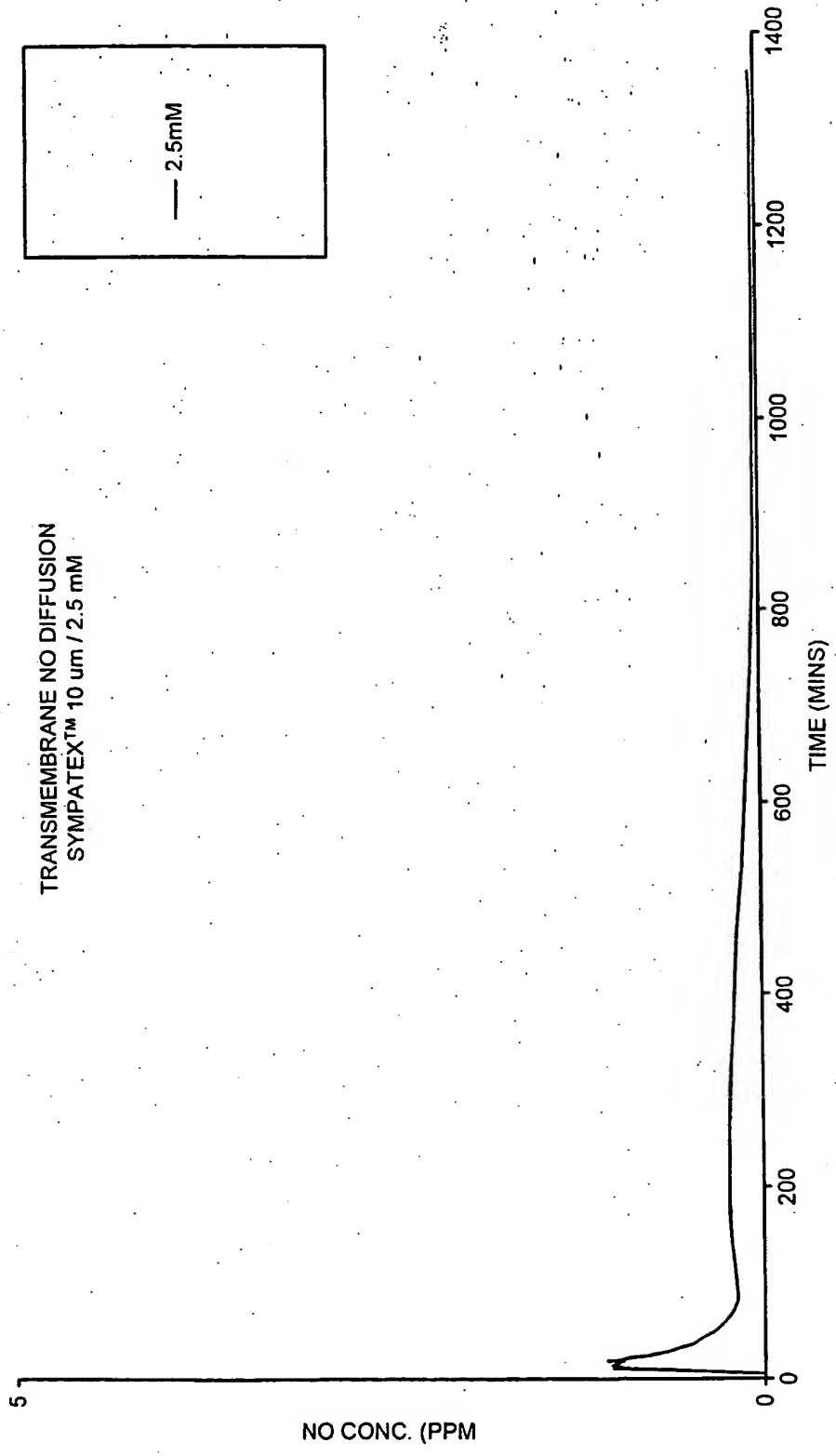
2.5mM
TRANSMEMBRANE NO DIFFUSION
SYMPATEX™ 10 um / 2.5 mM
5
0
NO CONC. (PPM
0
200
400
600
800
1000
1200
1400
TIME (MINS)

FIG. 6g

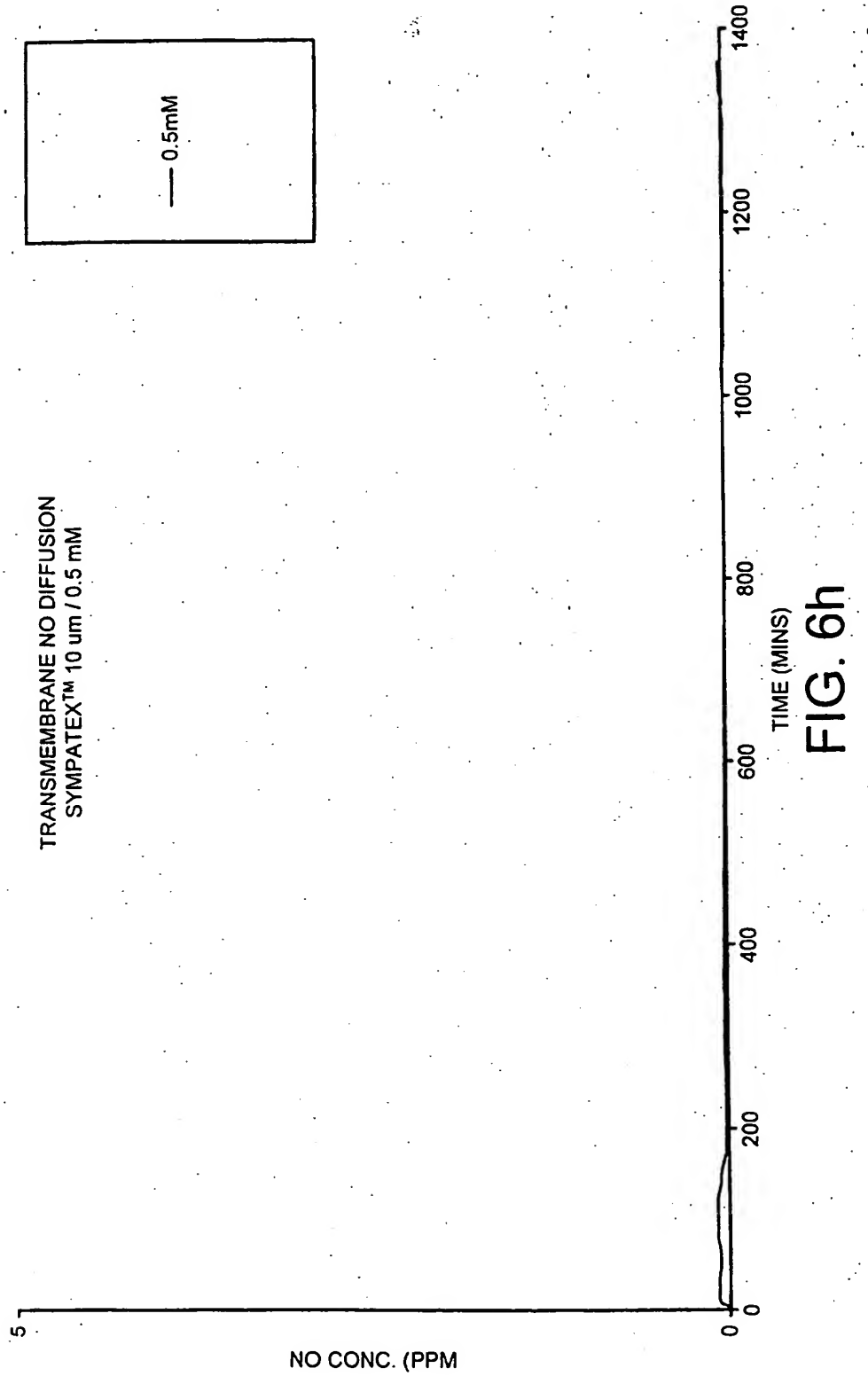
0.5mM
TRANSMEMBRANE NO DIFFUSION
SYMPATEX™ 10 um / 0.5 mM
TIME (MINS)
0
200
400
600
800
1000
1200
1400
NO CONC. (PPM
0
5

FIG. 6h

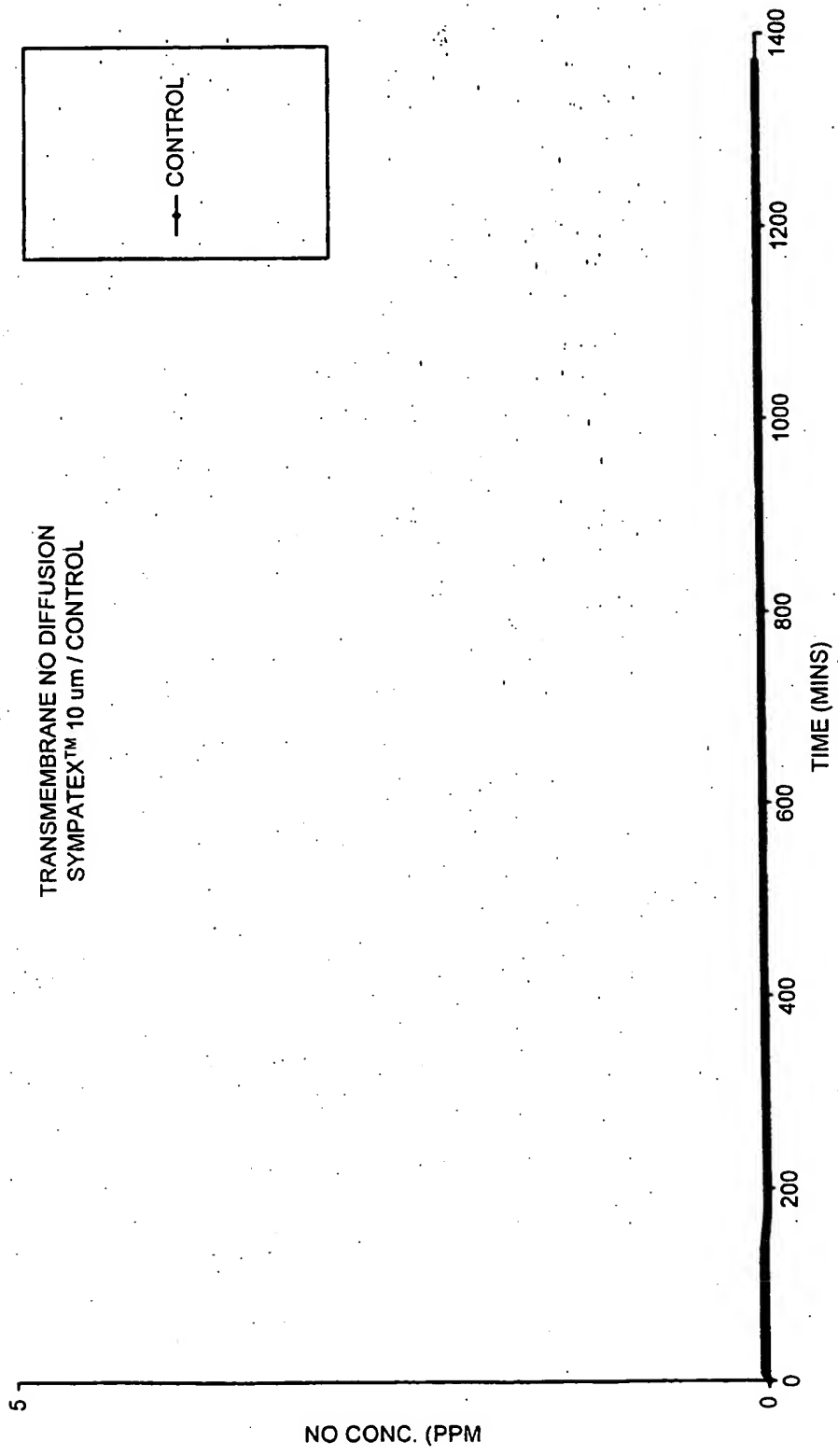
CONTROL
TRANSMEMBRANE NO DIFFUSION
SYMPATEX™ 10 um / CONTROL
TIME (MINS)
0
200
400
600
800
1000
1200
1400
NO CONC. (PPM
5
0

FIG. 6i

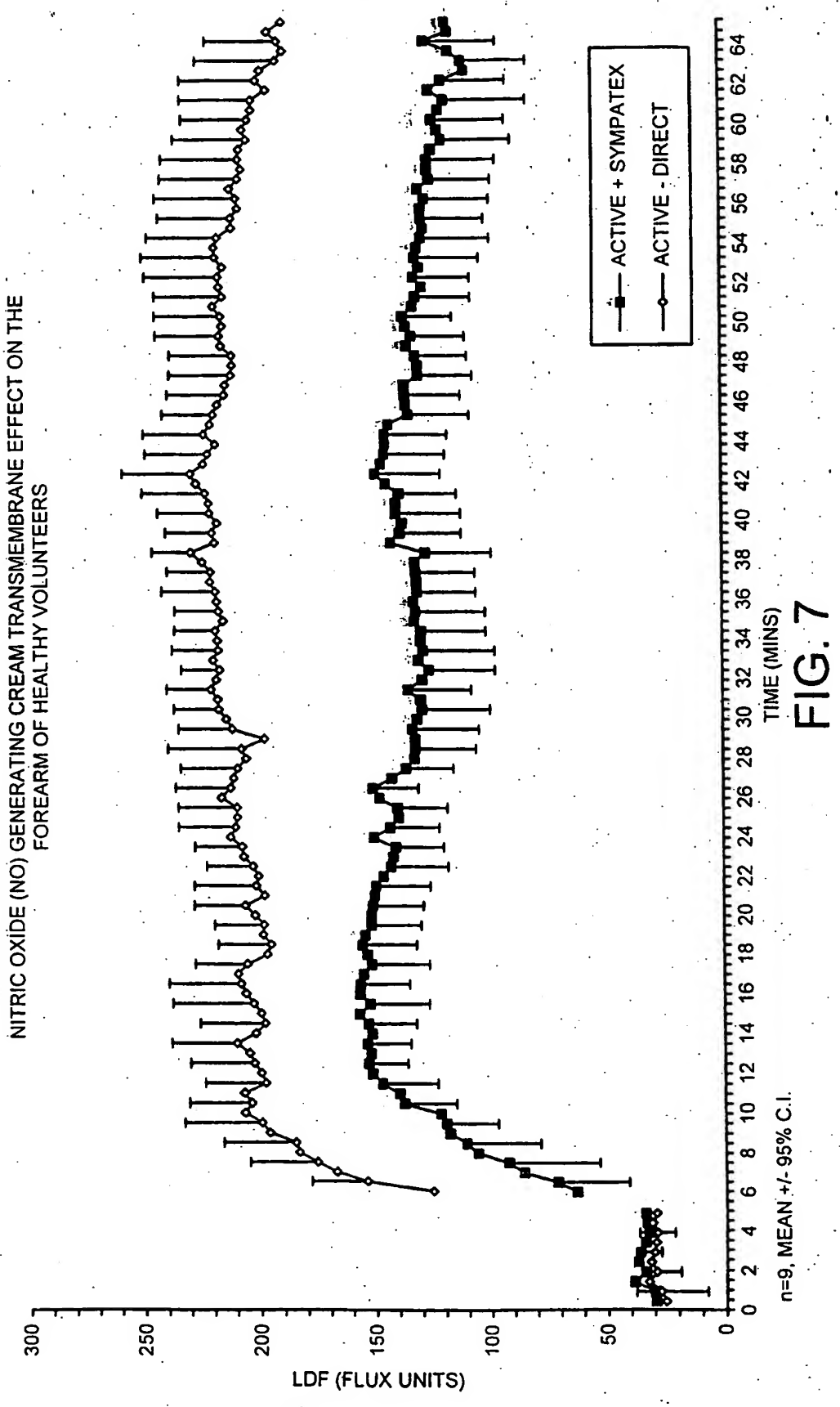
NITRIC OXIDE (NO) GENERATING CREAM TRANSMEMBRANE EFFECT ON THE FOREARM OF HEALTHY VOLUNTEERS
ACTIVE + SYMPATEX
ACTIVE - DIRECT
LDF (FLUX UNITS)
TIME (MINS)
n=9, MEAN +/- 95% C.I.

FIG. 7

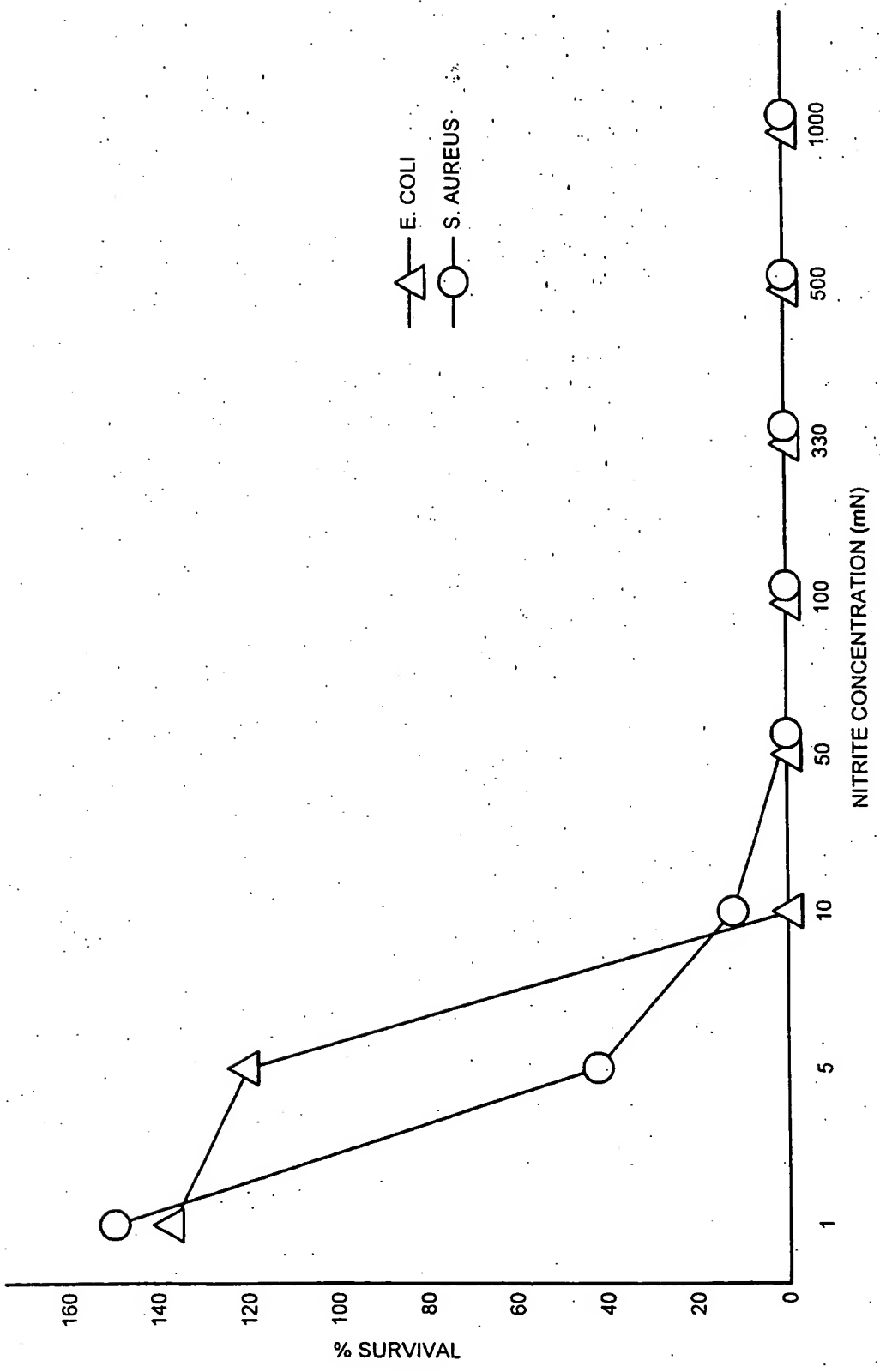
E. COLI
S. AUREUS
NITRITE CONCENTRATION (mN)
1000
500
330
100
50
10
5
1
% SURVIVAL
160
140
120
100
80
60
40
20
0

FIG. 8

INTERNATIONAL SEARCH REPORT

Inte. ..tional Application No
PCT/GB 00/00853

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K33/00 A61P17/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, PAJ, WPI Data, SCISEARCH, CHEM ABS Data, EMBASE, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 22335 A (UNIV ABERDEEN ;BENJAMIN NIGEL (GB); DOUGALL HAMISH (GB)) 24 August 1995 (1995-08-24) page 3, line 14 - line 26 page 4, line 11 - line 35 claims 1-5 ---	8-15,18
X	US 5 633 284 A (MEYER PETRUS JOHANNES) 27 May 1997 (1997-05-27) claims 1,2 examples 4,5 ---	1,2,6-17
X	US 5 648 101 A (TAWASHI RASHAD) 15 July 1997 (1997-07-15) examples 6-9 column 4, line 33 - line 67 claims 1-7 ---	1,2,4-16
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 August 2000	10/08/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Bonzano, C

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte Jonal Application No
PCT/GB 00/00853

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WELLER RICHARD ET AL: "A randomized trial of acidified nitrite cream in the treatment of tinea pedis." JOURNAL OF THE AMERICAN ACADEMY OF DERMATOLOGY, vol. 38, no. 4, April 1998 (1998-04), pages 559-563, XP002111730 ISSN: 0190-9622 page 559, column 2, paragraph 1 - paragraph 3 page 560, column 2, paragraph 2 ---	8-15,18
P,X	ORMEROD ANTHONY DAVID ET AL: "The inflammatory and cytotoxic effects of a nitric oxide releasing cream on normal skin." JOURNAL OF INVESTIGATIVE DERMATOLOGY, vol. 113, no. 3, September 1999 (1999-09), pages 392-397, XP000925553 ISSN: 0022-202X abstract page 393, column 1, paragraph 4 page 394, column 2, paragraph 3 ---	1-3,8-16
X	US 4 595 591 A (MARDI SHALVA ET AL) 17 June 1986 (1986-06-17) tables 1,2 column 5, paragraph 2 column 1 -----	1,4,6, 8-12

5

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1-18 relate to compounds defined (inter alia) by reference to the following parameters:

P1: acidifying agent.
P2: organic acid.
P3: pharmaceutically acceptable source of nitrite ions.
P4: nitrite precursor.
P5: associated conditions.

The use of these parameters in the present context is considered to lead to a lack of clarity within the meaning of Article 6 PCT. It is impossible to compare the parameters the applicant has chosen to employ with what is set out in the prior art. The lack of clarity is such as to render a meaningful complete search impossible. Consequently, the search has been restricted to the parts relating to the compounds mentioned in the description at pages 9-14 with due regard to the general idea underlying the present invention.
Claims searched completely: none.
Claims searched incompletely: 1-18.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 00/00853

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9522335 A	24-08-1995	AU 693851 B	09-07-1998
		AU 1669995 A	04-09-1995
		BR 9507099 A	16-09-1997
		CA 2183549 A	24-08-1995
		CN 1144484 A	05-03-1997
		CZ 9602456 A	15-01-1997
		EP 0746327 A	11-12-1996
		FI 963232 A	12-09-1996
		JP 9508921 T	09-09-1997
		NO 963446 A	21-10-1996
		PL 316033 A	23-12-1996
US 5633284 A	27-05-1997	AU 667549 B	28-03-1996
		AU 4322593 A	04-01-1994
		DE 69324011 D	22-04-1999
		DE 69324011 T	07-10-1999
		EP 0644766 A	29-03-1995
		GR 3030525 T	29-10-1999
		JP 8500092 T	09-01-1996
		NO 944719 A	07-12-1994
		AT 177642 T	15-04-1999
		CA 2136614 A	23-12-1993
		WO 9325213 A	23-12-1993
		ES 2132236 T	16-08-1999
		NZ 252895 A	28-10-1996
		ZA 9303895 A	27-12-1993
US 5648101 A	15-07-1997	NONE	
US 4595591 A	17-06-1986	CH 629100 A	15-04-1982
		AR 224654 A	30-12-1981
		AT 20183 T	15-06-1986
		AU 538878 B	30-08-1984
		AU 6275880 A	09-04-1981
		CA 1155058 A	11-10-1983
		DE 3071629 D	10-07-1986
		DK 407480 A,B,	28-03-1981
		EG 15021 A	30-06-1985
		EP 0026532 A	08-04-1981
		ES 495330 D	01-10-1981
		ES 8107020 A	16-12-1981
		FI 803037 A,B,	28-03-1981
		GR 70008 A	23-07-1982
		IL 61136 A	29-02-1984
		IN 154851 A	15-12-1984
		JP 1029773 B	14-06-1989
		JP 1546372 C	28-02-1990
		JP 56059706 A	23-05-1981
		KR 8501724 B	07-12-1985
		PH 18810 A	27-09-1985
		PL 226939 A	21-08-1981
		SU 1382394 A	15-03-1988
		ZA 8005990 A	30-09-1981